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Cells from Without: Leukocytes, Surface Proteins, and the Strategy of Gating

In this chapter, the analysis of leukocytes, one of the more important applications of flow cytometry, is discussed. It is an important application not because leukocytes are intrinsically more important than other types of cells but because they constitute a class of particles that, for several reasons, are ideally suited for analysis by flow cytometry and thus make very good use of the capabilities of the technique. Leukocytes therefore account for a large proportion of the material analyzed by flow cytometry in both hospital and research laboratories. They also serve as good examples for describing here some of the general procedures of cytometric protocol, particularly the art of gating and the requirements for controls.

Leukocytes are well suited to flow analysis for three reasons. First, they occur in vivo as single cells in suspension; they can therefore be analyzed without disaggregation from a tissue mass (and no spatial information is lost in the course of preparation). Second, the normal suspension of leukocytes (i.e., blood) contains a mixture of cell types that happen to give off forward (FSC) and side (SSC) light scatter signals of different intensities, thereby allowing them to be distinguished from each other by flow cytometric light scatter parameters. Third, with monoclonal antibody technology, a vast array of specific stains for probing leukocyte surface proteins has been developed; these stains have allowed the classification of microscopically identical cells into various subpopulations according to staining characteristics that are distinguishable by means of flow analysis.

I will first describe leukocytes in a general way for those not familiar with this family of particles. We will then proceed to a description of general staining techniques for surface markers, with attention to the need for controls. At this point I will digress slightly and discuss the way cytometry results can (and cannot) be quantified and the way sensitivity can affect these results. Finally, I will discuss gating strategies, in both philosophy and practice.

CELLS FROM BLOOD

Whole blood consists of cells in suspension (Fig. 6.1). In a “normal” milliliter (cm^3) of blood, there are about 5×10^9 erythrocytes (red blood cells), which are shaped like flat disks (about $8 \mu\text{m}$ in diameter) and are responsible for oxygen transport around the body. In addition, per cm^3 there are about 7×10^6 white cells (leukocytes) that collectively are involved in immune responses in the organism. The leukocytes appear to be heterogeneous under the microscope and can be divided according to their anatomy into several classes. There are cells that are called *monocytes*, which are of a concentration of about 0.5×10^6 per cm^3 ; these appear on slides as round cells (about $12 \mu\text{m}$) with horseshoe-shaped nuclei and are responsible for phagocytosis of invading organisms and for presenting antigens in a way that can initiate the immune response.

There are also cells that appear as $10 \mu\text{m}$ circles with large round nuclei and a narrow rim of cytoplasm; these are the *lymphocytes*, present in a concentration of about 2×10^6 per cm^3 . Lymphocytes (including both B lymphocytes and T lymphocytes) are responsible for a great variety of the functions that are known as *cellular* and *antibody* (humoral) *immunity*, as well as for the regulation of those functions. The third (and most frequent) group of leukocytes occurs

Fig. 6.1. Scanning electron micrographs showing the different surface textures of red (Er) and white blood cells. **A:** Cells within a blood vessel. **B,C:** A comparison of scanning electron micrographs with conventional light microscope images of the same field of stained cells. Enlarged pictures at the right emphasize the different surface textures of monocytes (Mo) and platelets (Pl) in **D**, lymphocytes (Ly) in **E**, and neutrophils (Ne) in **F**. From Kessel RG and Kardon RH (1979). *Tissues and Organs: A Text Atlas of Scanning Electron Microscopy*, WH Freeman, NY.

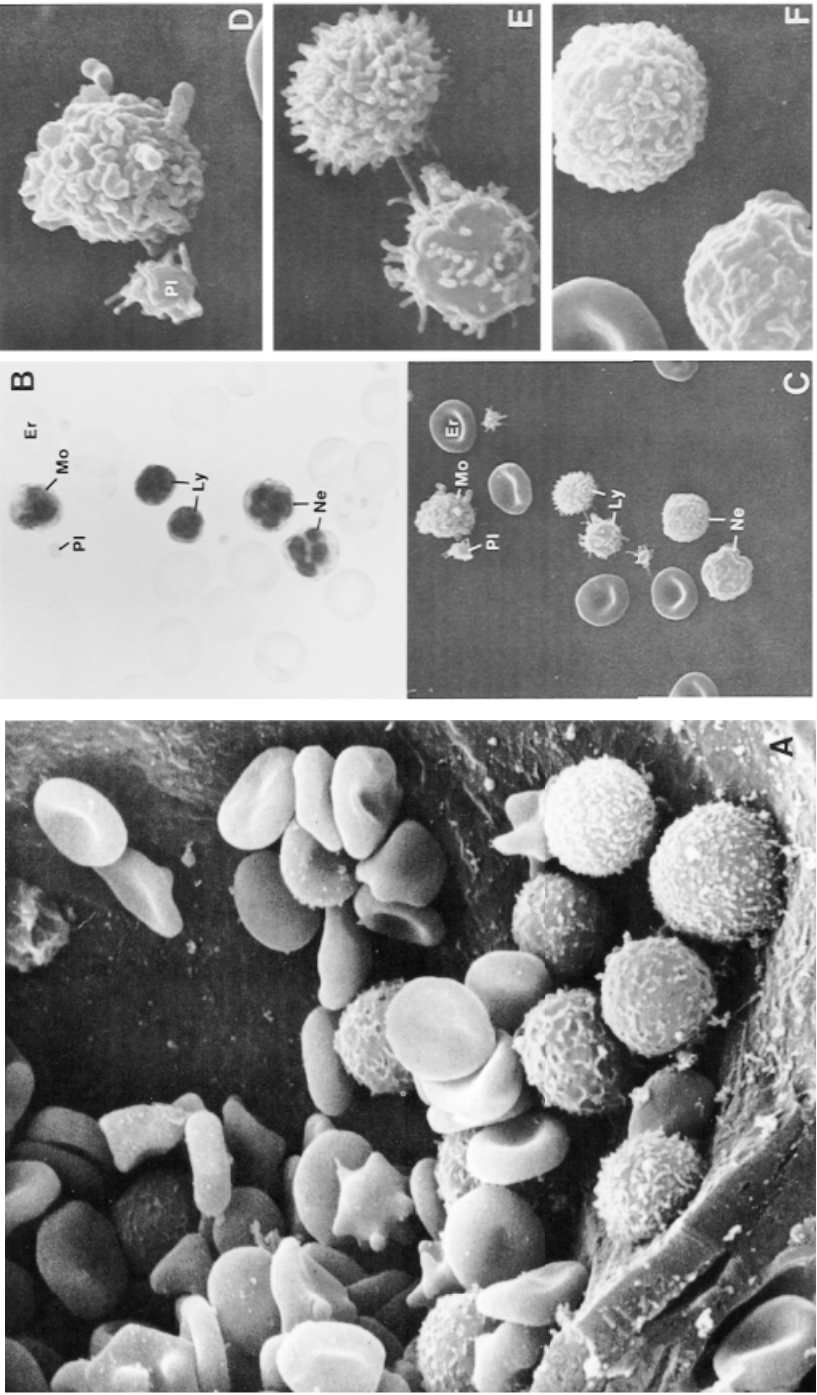


TABLE 6.1. Cells in Normal Human Adult Peripheral Blood

Cells	No. per cm ³ (95% range)	Percent of WBC	Diameter (μm)
Platelets (thrombocytes)	$1-3 \times 10^8$		2-3
Erythrocytes (RBC)	$4-6 \times 10^9$		6-8
Leukocytes (WBC)	$3-10 \times 10^6$	[100]	
Granulocytes			
Neutrophils	$2-7 \times 10^6$	50-70	10-12
Eosinophils	$0.01-0.5 \times 10^6$	1-3	10-12
Basophils	$0-0.1 \times 10^6$	0-1	8-10
Lymphocytes	$1-4 \times 10^6$	20-40	6-12
Monocytes	$0.2-1.0 \times 10^6$	1-6	12-15

Values are from Lentner C, ed. (1984). Geigy Scientific Tables, 8th edition. CIBA-Geigy, Basle; and from Diggs LW, et al. (1970). The Morphology of Human Blood Cells, 5th edition. Abbott Laboratories, Abbott Park, IL.

in a concentration of about 5×10^6 per cm³. They are distinguished under the microscope by lobular nuclei and an array of cytoplasmic granules, are therefore called *polymorphonuclear cells* or *granulocytes* (including basophils and eosinophils, but mainly neutrophils), and are collectively responsible for phagocytic as well as other immune-related activities. In addition, there are small, cell-derived particles called *platelets* (about 2 μm in size), in a concentration of about 2×10^8 per cm³. The platelets are involved in mechanisms for blood coagulation (Table 6.1).

Although the blood is an easily accessible tissue to study, because red blood cells outnumber leukocytes by about 1000 to 1 in the peripheral circulation, the analysis of leukocytes by any technique is difficult unless the red cells can be removed. Techniques for removing red cells usually involve either density gradient centrifugation (pelletting red cells and neutrophils, leaving lymphocytes and monocytes behind to be collected from a layer as a peripheral blood mononuclear cell preparation [PBMC]) or differential lysis of red blood cells, leaving intact the more robust lymphocytes, monocytes, and neutrophils.

The light scatter signals (FSC and SSC) resulting from flow cytometric analysis of whole blood, lysed whole blood, and a mononuclear cell preparation after the density gradient separation of whole blood are compared in Figure 6.2. Each dot plot shows 2000 cells;

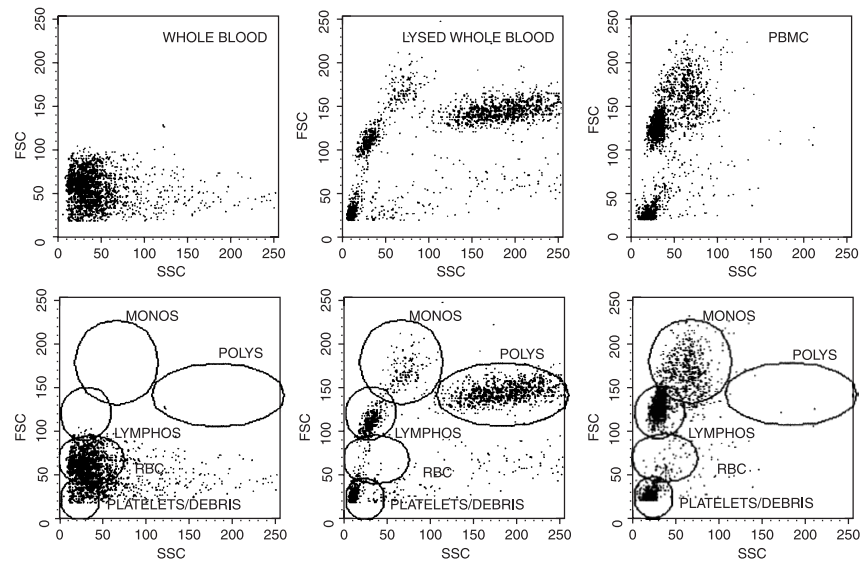


Fig. 6.2. The FSC and SSC signals resulting from the cells in different blood preparations (whole peripheral blood; whole peripheral blood after erythrocyte lysis; and peripheral blood mononuclear cells [PBMCs] with granulocytes removed by density gradient centrifugation). The bottom panels indicate the five clusters into which the scatter signals fall.

each cell is represented by a dot and plotted on the two axes according to the intensity of its FSC and SSC signals. The resulting clusters of particles consist of

1. A group of particles with low SSC intensity and low FSC intensity (and usually ignored because they fall below the useful FSC threshold)
2. A group of particles with low SSC intensity but somewhat higher FSC intensity
3. A group of particles, still with low SSC intensity but with moderately high FSC intensity
4. A group of particles with somewhat higher FSC but also with moderate SSC intensity
5. A group of cells possessing moderate FSC but much higher SSC.

The most obvious way to determine the identity of these particles, with distinctive scatter characteristics, is to sort them with a sorting flow cytometer (see Chapter 9). The flow sorting of blood preparations according to FSC and SSC parameters has brought the realization that the clusters of particles with defined flow cytometric scatter characteristics belong to the groups of cells as distinguished, traditionally, by microscopic anatomy. The anatomical differences that we see under the microscope result from different patterns of light bouncing off the cells on the slide and being registered by our eyes. Therefore, it should not be entirely surprising that a flow cytometer, with its photodetectors measuring light bouncing off cells, often clusters cells into groups that are familiar to microscopists. In addition, however, the human eye registers many more than two parameters when it looks at a cell (think of shape, texture, movement, color). Specifically, the eye registers something we might call *pattern* very sensitively; a flow cytometer registers pattern not at all. So, in some ways, it is perhaps just good luck that the two parameters of FSC and SSC light do allow cytometrists to distinguish some of the classes of white cells almost as effectively as a trained microscopist with a good microscope.

We should, however, always be aware of situations in which microscopists are better than cytometrists—certain types of classification are easy by eye but not at all easy by cytometer. For example, a microscopist would never confuse a dead lymphocyte with an erythrocyte, nor a chunk of debris with a viable cell; such mistakes are all too frequent in cytometry. Furthermore, microscopists, if they were not so polite, would find it laughable that cytometrists have a great deal of difficulty in distinguishing clumps of small cells from single large cells. However, if we think back to the discussion in Chapter 3 about the origin of the FSC signal, we can see why these problems occur and why we need to be aware of the limits of flow cytometry (and why a microscope is an essential piece of equipment in a flow cytometry lab).

The patterns of light scatter distribution illustrated in Figure 6.2 result from analysis of blood from a normal individual. With patterns like this, a flow cytometrist can, with some practice, set a so-called lymphocyte region around a group of particles that are mainly lymphocytes. This lymphocyte region will then, using FSC and SSC char-

acteristics, define the cells that are to be selected (gated) for analysis of fluorescence staining. Such lymphocyte gating is the direct equivalent of a skilled microscopist's decision, based on size and nuclear shape, about which cells to include as lymphocytes in a count on a slide. A detailed discussion of gating is given at the end of this chapter. Because staining can provide us with some help in our gating decisions, we need first to discuss some of the staining techniques that can help us to describe cell populations.

STAINING FOR SURFACE MARKERS

Monoclonal antibody technology has provided flow cytometrists with a large array of antibodies that are specific for various proteins on the leukocyte surface membrane. The proteins (antigens), defined by these antibodies, have been given so-called CD numbers; "CD" stands for "cluster of differentiation" and refers to the group or cluster of antibodies all of which define a particular protein that differentiates cells of one type. More and more antigens are defined each year, and the CD numbers now range from CD1 on up through CD170 or greater. Antibodies against these antigens (called anti-CDx antibodies) have allowed immunologists to define taxonomic subgroups of leukocytes that are microscopically indistinguishable from each other but whose concentrations vary in ways that are related to an individual's immune status. For example, B lymphocytes and T lymphocytes look identical under the microscope and have similar FSC and SSC characteristics on the flow cytometer, but they possess membrane proteins that allow them to be distinguished by antibody staining (for example, B lymphocytes have CD20 on their surface; T lymphocytes have CD3). Some of the membrane antigens on leukocytes define function, some define lineage, some define developmental stage, and some define aspects of cell membrane structure whose significance is not yet understood. Although in this chapter I discuss the staining of leukocyte surface antigens with monoclonal antibodies, the principles apply equally well to the use of antibodies for staining any surface antigens on any type of cell.

An antibody will form a strong bond to its corresponding antigen.

To be of use in microscopy or flow cytometry, this bond needs to be “visualized” (to the eye or to the photodetector) by the addition of a fluorescent tag. Visualization can be accomplished by one of two different methods. With direct staining, cells are incubated with a monoclonal antibody that has been previously conjugated to a fluorochrome (for example, fluorescein or phycoerythrin or any fluorochrome with appropriate absorption and emission spectra). This procedure is quick and direct; it merely involves a half-hour incubation of cells with antibody (at 4°C), followed by several washes to remove weakly or nonspecifically bound antibodies. Cells thus treated are ready for flow analysis (although final fixation with 1% electron microscopic-grade formaldehyde will provide a measure of biological safety and long-term stability).

The second method (indirect staining) is more time consuming, less expensive, and either more or less adaptable depending on the application. Indirect staining involves incubation of cells with a non-fluorescent monoclonal antibody, washing to remove weakly bound antibody, and then a second incubation with a fluorescent antibody (the so-called second layer) that will react with the general class of monoclonal antibody used in the first layer. For example, if the primary monoclonal antibody happens to be an antibody that was raised in a mouse hybridoma line, it will have the general characteristics of mouse immunoglobulin; the second layer antibody can then be a fluoresceinated (fluorescein-conjugated) antibody that will react with any mouse immunoglobulin. The advantages of this two-layer technique are that, first, monoclonal (primary layer) antibodies are cheaper if they are unconjugated; second, a given second layer reagent can be used to visualize any monoclonal antibody of a given class (e.g., any mouse immunoglobulin, in our example here); third, comparison of antigen density according to fluorescence intensity is easier if common second layer reagents are used; and fourth, each step in the staining procedure results in amplification of the fluorescence intensity of the staining reaction. (With regard to this signal amplification that occurs with indirect staining, I might also add parenthetically that further amplification of weak signals is possible by using third and fourth layer reagents of appropriate specificity. All that is required is an interest in zoology. For example, if the primary monoclonal antibody is a mouse monoclonal, the second layer reagent could be a fluorescein-conjugated antibody raised in a goat and

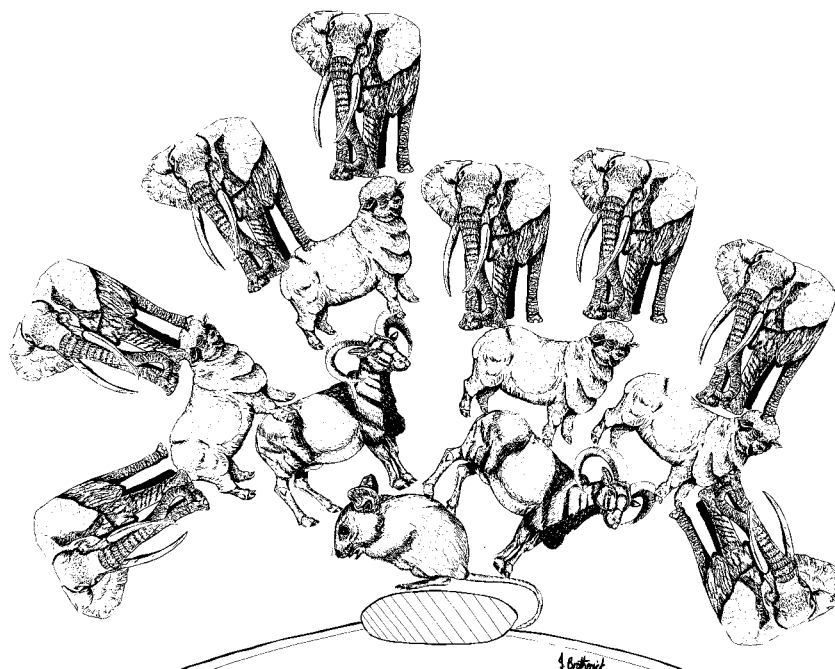


Fig. 6.3. Amplification of staining by the use of multiple antibody reagents. Drawing by Ian Brotherick.

specific for mouse Ig [known as a *goat anti-mouse reagent*]. An appropriate third layer reagent might then be a fluorescein-conjugated antibody raised in a sheep and specific for goat Ig [sheep anti-goat], and so on [elephant anti-sheep, armadillo anti-elephant, unicorn anti-armadillo] until the zoologists run out of immunologically competent animals [Fig. 6.3]. Because each antibody molecule is linked to many fluorochrome molecules and because many antibodies will bind to each antigen, this is a way to increase the intensity of signals from sparsely expressed membrane proteins.)

The disadvantages of indirect staining are that it is more time-consuming and it involves a second step that doubles the opportunity for nonspecific binding. Indirect staining also greatly limits the opportunity for simultaneous double and triple staining of cells with two and three different fluorochromes because of the problems of

cross-reactivity between primary antigens and the conjugated second layer reagents that may display broad specificity. Nevertheless, with appropriate choice of monoclonal antibodies of specific immunoglobulin subclass and/or animal derivation and with second layer reagents appropriate and specific to these particular characteristics, two-color staining with indirect reagents is sometimes possible—but it is not easy. In general, with the increasing availability of multilaser systems and the concurrent realization by scientists of the informative power of multicolor staining, most workers have adopted direct staining procedures.

CONTROLS

As emphasized in the section in Chapter 3 on electronics, the intensity “read out” in flow cytometry is relative and user-adjustable. By changing electronic settings, cells of a given intensity can receive either high or low “intensity” values from the ADC (and can be placed at high or low positions on the fluorescence scale). Therefore, in order to know whether cells that have been exposed to a stain have actually bound any of that stain, we need to compare the stained cells with an unstained control. One of the general laws of science that applies particularly to flow cytometry is that no matter how many controls you have used in an experiment, when you come to analyze your results you always wish you had used one more. There are three reasons that this problem is acute in flow analysis. One has to do with the background fluorescence of unstained cells; the second has to do with the nature of antibody–antigen interactions; and the third has to do with the problem of compensation between overlapping fluorescence spectra from different fluorochromes.

The first problem that needs to be controlled is that of background fluorescence (called *autofluorescence*). All unstained cells give off some fluorescence (that is, all cells emit some light that gets through one or another of the filters in front of a cytometer’s photodetectors). This autofluorescence may not be recognized by microscopists either because it is very dim or because experienced microscopists have acquired a mental threshold in the course of their training. But our cytometer’s photodetectors are both very sensitive and completely untrainable. Therefore the autofluorescence of cells,

resulting from intracellular constituents such as flavins and pyridine nucleotides, is bright enough to be detected. It can, in some cells, be so bright as to limit our ability to detect positive staining over and above this bright background. Whatever the level of this autofluorescence, we need to define it carefully by analyzing unstained cells (autofluorescence controls) if we are going to be able to conclude that cells treated with a reagent have actually become stained (that is, are now brighter than their endogenous background).

Beyond the problem of autofluorescence, there is a second problem. As discussed above, much of the staining of cells for flow analysis makes use of antibody–antigen specificity. Although the specificity between an antibody's binding site (the key) and the corresponding epitope on an antigen (the lock) is indeed exquisite, the beauty of the system can be confounded by a long floppy arm on the back (Fc) end of the antibody. These Fc ends stick with wild abandon to so-called Fc receptors that occur on the surface of many types of cells (notoriously monocytes). While I have worked in a department with scientists who study the specificity and importance of this Fc binding for too long to consider these reactions to be nonspecific and only a nuisance, it is true that these Fc receptors on many types of cells can confound the nominal specificity of an antibody's binding to its reciprocal antigen. What this means is that cells may stain with a particular monoclonal antibody because they possess a particular antigen on their surface membrane that locks neatly with the key on the monoclonal antibody binding site. They may also, however, stain with that particular monoclonal antibody because they possess Fc receptors that promiscuously cling to antibodies with all antigenic specificities. In addition, it is often true that dead cells (with perforated outer membranes) can soak up antibodies and then hang on to them tenaciously. The way to know if staining of cells is specific to a specific antigen is to use the correct control.

The correct control is always an antibody of exactly the same properties as the monoclonal antibody used in the experiment, but with an irrelevant specificity. If we are staining cells with a monoclonal antibody having a specificity for the CD3 protein occurring on the surface of T lymphocytes (and that monoclonal antibody happens to be a mouse immunoglobulin of the IgG_{2a} subclass, conjugated with six fluorescein molecules per molecule of protein and used to stain the cells at a concentration of 10 µg per ml), then an appropri-

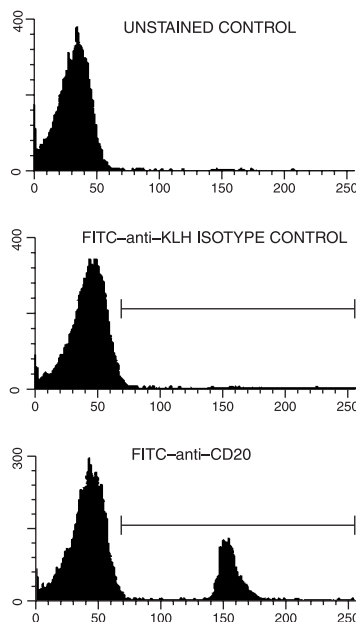


Fig. 6.4. The fluorescence histogram of an isotype control sample is used to decide on the fluorescence intensity that indicates positive staining.

ate control would be a mouse monoclonal antibody of the same subclass, with the same fluorescein conjugation ratio, and at the same protein concentration, but with a specificity for something like key-hole limpet hemocyanin or anything else that is unlikely to be found on a human blood cell (Fig. 6.4).

Such a control antibody is known as an *isotype control* because it is of the same immunoglobulin isotype (subclass) as the staining antibody used in the experiment. It will allow you to determine how much background stain is due to irrelevant stickiness (dead cells, Fc receptors, and so forth). The only trouble with this scenario is that exactly correct isotype controls are not usually available. Various manufacturers of monoclonal antibodies will sell so-called isotype controls and will certainly recommend that they be used. These are, however, general purpose isotype controls that will be of an average

fluorochrome conjugation ratio and of a protein concentration that may or may not be similar to that used for most staining procedures. Whether an average isotype control is better than no isotype control at all is a matter of opinion and will depend on the kinds of answers that you demand from your experiments. For most routine immunophenotyping, where the staining of positive cells is strong and bright, isotype controls have been falling out of favor. Unstained (autofluorescence) controls may be good enough.

The third problem that needs to be controlled is that of spectral cross-over and the possibility of incorrect instrument compensation. As an example of a case in which controls for nonspecific staining, autofluorescence, and compensation are all critical, let us look at the staining of B lymphocytes for the CD5 marker present with only low density on their surface. As well as the problems created by nonspecific staining and by autofluorescence, the problem of spectral cross-over between fluorescein and phycoerythrin can particularly confuse the interpretation of results from this kind of experiment. Look at Figure 6.5. What we are interested in is the number of B lymphocytes that possess the CD5 surface antigen. These cells will appear in quadrant 2 of a contour plot of fluorescein fluorescence

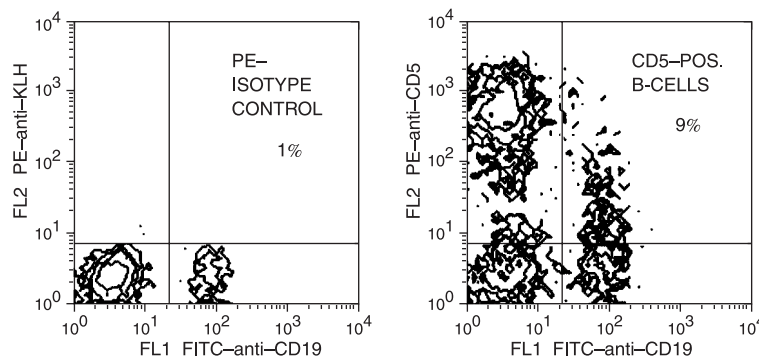


Fig. 6.5. The use of a phycoerythrin (PE) isotype control to help in deciding where, in a dual-color plot, to draw the horizontal line between fluorescein-stained cells to be considered positive and those to be considered negative for the PE stain. Misplacing of the horizontal line will affect the number of CD19 cells determined to express the CD5 antigen in the stained sample. Data courtesy of Jane Calvert.

(a B-lymphocyte stain) on the horizontal axis against phycoerythrin (PE) fluorescence (the CD5 stain) on the vertical axis. But B cells will also appear in this quadrant if they have orange autofluorescence or if they are nonspecifically sticky for the anti-CD5 antibody (in this case a mouse monoclonal immunoglobulin of the IgG_{2a} isotype). In addition, they will appear in this quadrant if the cytometer's orange photodetector has not been properly compensated for cross-over from the fluorescein signal. The way around all these problems is to stain cells with a fluorescein stain for B cells in conjunction with an isotype control (a mouse IgG_{2a} antibody conjugated with PE but specific for an irrelevant antigen, say, keyhole limpet hemocyanin). The intensity of stain shown by these control cells on the PE photodetector will mark the limit of intensity expected from all nonspecific causes. Any further PE intensity shown by cells stained with the B-cell stain and the anti-CD5 PE stain will now clearly be the result of specific CD5 proteins on the cell surface. In this way, by use of the correct isotype control, we can rule out any problems in interpretation that may result from incorrect instrument compensation or nonspecific or background fluorescence.

In general, all these problems and their appropriate controls are particularly important when, as with the CD5 antigen on B cells, the staining density on the cells in question is low and there is considerable overlap between positive and negative populations. They become less critical for the evaluation of results when dull negative cells are being compared with a bright positive population. In any case, the general procedure for analyzing flow data is to look at the level of background staining (resulting from both autofluorescence and nonspecific staining) and then, having defined this intensity, to analyze the change in intensity that occurs after the cells have been stained. As discussed in Chapter 4, this change may consist of the bright staining of a small subpopulation within the total population; in this situation, the relevant result may be given as the percentage of the total number of cells that are positively stained. Alternatively, the change may involve the shift of the entire population to a somewhat brighter fluorescence intensity; here the relevant result may be expressed as the change in brightness (mode, mean, or median of the distribution). This leads us to the problem of quantification of intensity by flow cytometry.

QUANTITATION

One of the proclaimed advantages of flow cytometry, compared with eyeball microscopy, is its quantitative nature. Flow cytometry is indeed impressively quantitative when it comes to counting cells and compiling statistics about large numbers of cells in a short period of time. Users are, however, subjected to a rude shock when they first attempt to quantify the fluorescence *intensity* of their cells. Whereas a flow cytometer can be very quantitative about *comparing* the fluorescence intensity of particles (assuming that the photodetectors and amplifiers are working well), it is unfortunately true that a flow cytometer is very bad at providing an absolute value for the light intensity it measures. Therefore, any experimental protocol that needs to measure the intensity of the staining of cells (as opposed to a yes or no answer about whether and what percentage of cells are stained or not) is up against certain intrinsic difficulties.

If you really do need some measure of the intensity of cells, the way around these difficulties is to accept the limitations of the system, work within the constraints, and use some kind of standard to calibrate the intensity scale. The easiest standard for any cell is its own unstained control. An arbitrary position on the scale can be assigned to the fluorescence of the control (by changing the voltage on the photomultiplier tube during instrument set-up), and the stained sample can be compared with this. The disadvantage in this measure of relative fluorescence compared with the control is that cells with high autofluorescence will require a greater density of positively stained receptors to give the same “relative intensity” value as cells with low autofluorescence. In other words, if intensity is expressed by a ratio of the brightness of stained cells relative to that of the unstained cells, a given ratio will represent more positive stain (in terms of fluorochrome molecules) on highly autofluorescent cells than on cells with low background.

One way around this problem is to compare cell fluorescence not with unstained cells but with the fluorescence of an external standard. This can be done by the use of fluorescent beads. In brief (this is an insiders’ joke; you would be amazed at how much has been written about the use of beads in flow cytometry), there are commercially available polystyrene beads (“microspheres”) that have standardized

fluorescence intensities. Some of these beads have fluorescein or PE bound to their surface. Others have a selection of hydrophobic fluorochromes incorporated throughout the bead. The latter are more stable in intensity, but, because the fluorochromes are not the usual flow cytometric fluorochromes, they may give different relative values on the different photodetectors of different cytometers. In either case, by running a sample of standard beads through the cytometer, all data can be reported as a value relative to the intensity of the standard beads.

One further step toward calibration has been taken with the use of a calibration curve made with sets of beads with known numbers of fluorochromes on their surface. Such calibrated beads are available with known numbers of PE molecules. Similar, but less direct, beads are available with fluorochrome molecules that have been calibrated in units equivalent to the intensity of fluorochrome molecules in solution ("MESF" units = "molecular equivalents of soluble fluorochrome"). With these beads, a curve can be obtained (Fig. 6.6), giving each channel on the ADC a calibration in number of fluorochrome molecules (for PE) or MESF values (for fluorescein). In this way, the background fluorescence of a control sample can be expressed as an equivalent number of fluorochrome (or MESF) molecules and can be subtracted from the number of fluorochrome molecules of a stained sample. The fluorescence of the stained sample can then be expressed as, for example, PE molecules over and above the background level.

Having now determined a value that might, with luck, quantify the brightness of a particle in terms of fluorochrome molecules or soluble equivalents, one may wonder how best to convert that value into the number of receptors or antigens on the surface of the cell. At first thought, calculation of this value might be determined if values are known for the number of fluorochrome molecules per antibody (the F/P ratio) used in the staining procedure. Unfortunately, even if this value has been determined chemically, it will not apply within a system in which there is quenching of the fluorescence from fluorochromes in closely packed regions on a cell surface (causing a bound fluorochrome to fluoresce considerably less brightly than in its soluble form). Moreover, the F/P value will almost certainly not be known in a system with indirect staining and undetermined amplification. At the present time, this F/P value can only be used with confidence in certain staining systems where antibodies have been certified to contain a single PE molecule per antibody and are used in

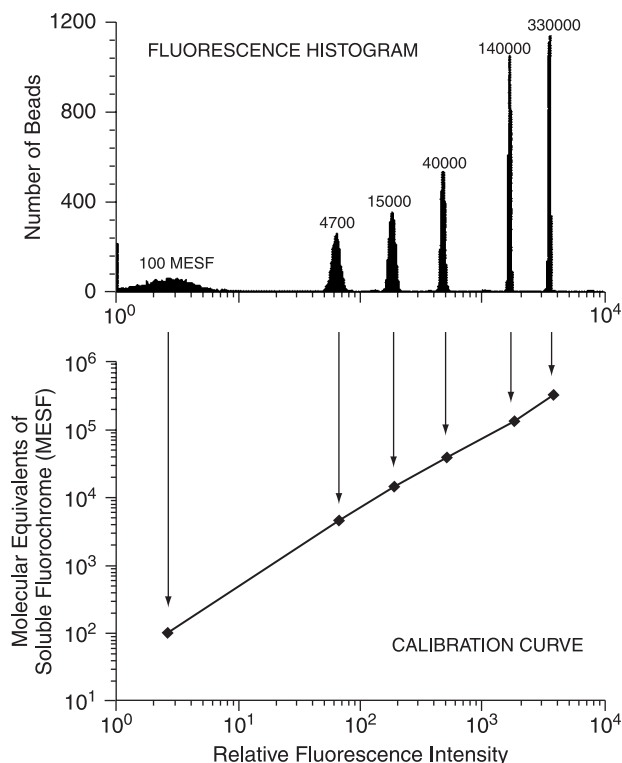


Fig. 6.6. The fluorescence histogram of a mixture of fluorochrome-conjugated calibration beads and the calibration line for channel numbers and their equivalence in soluble fluorescein molecules derived from that histogram. From Givan (2001).

conjunction with standard beads with known numbers of PE molecules. Even in this case, the final value will be in terms of the number of antibodies bound to a cell, and this may not be easily related to the number of receptors per cell (because antibody binding to receptors may be monovalent or bivalent).

Other types of calibration help may be available in the form of a different type of calibrated microsphere. Beads can be obtained that possess a known number of binding sites for immunoglobulin molecules. They can be treated as if they were cells and stained in the routine way with the antibody stain (direct or indirect) in question. The intensity of the beads with known numbers of antibody binding sites can be used to calibrate the scale, converting ADC channels to

antibody binding sites per cell. Problems with quantification using these beads derive from the fact that the avidity of the beads for antibodies can differ from the avidity of cells for antibodies so that receptors on beads and on cells may not saturate at equivalent concentrations. In addition, as above, antibodies have the possibility of binding either monovalently or bivalently under different conditions.

SENSITIVITY

Light detection sensitivity for stained cells is determined by two factors: the amount of background signal from the cells and the breadth of the population distributions of the background and of the positive signals that you are trying to detect over background. In other words, you can detect staining on cells that is just slightly brighter than background if none of the stained cells overlaps with the brightest cells in the unstained (control) population. However, if the control and fluorescent populations have very broad distributions (that is, the range of values is great), their averages have to be well separated from each other if you are going to be able to say that a particular cell belongs to a stained population rather than a background population (Fig. 6.7). This is, in concept, no different from the requirement in statistics for a narrow standard deviation to conclude that two populations with closely similar means are significantly different from each other, but a less stringent requirement for narrow standard deviation if the two population means are well separated.

As a practical matter, the way to make a flow cytometer more sensitive in detecting weak light signals is to lower the noise in the background by using good optical, fluidic, and electronic components and to align the instrument well so that fluorescence detection efficiency is high and fluorescence distributions are as narrow as possible. The result of these considerations leads, however, to the unavoidable conclusion that cells with intrinsically high background make it more difficult to detect low numbers of fluorochromes derived from the staining procedure. Think of trying to see the stars in the daytime. Certain classes of cells have greater autofluorescence than others as a result of their metabolic activity. All other things being equal, large cells have more autofluorescence than small cells, simply because they are larger and have more autofluorescent molecules associated with each cell.

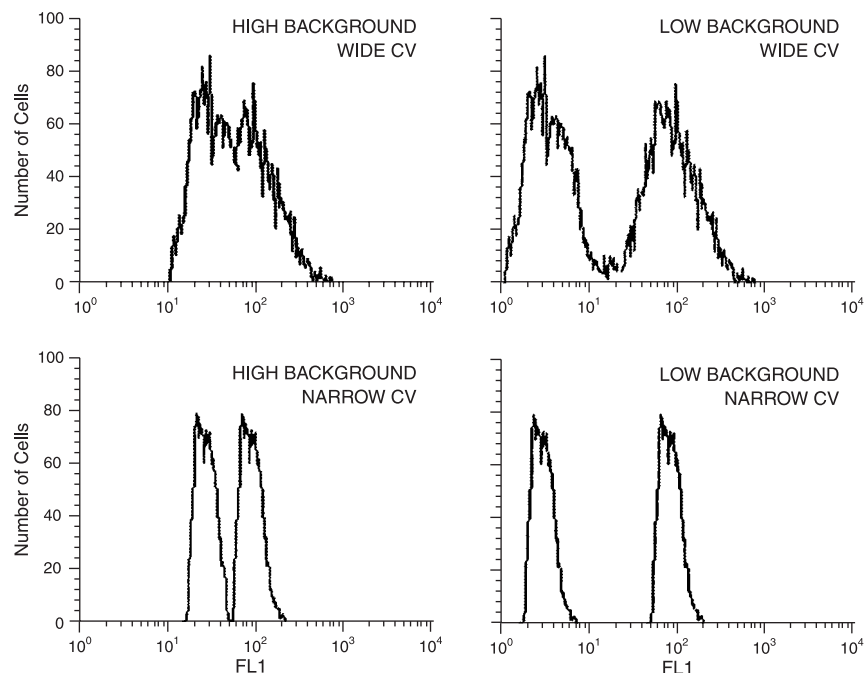


Fig. 6.7. The ability to distinguish stained cells from unstained cells depends on both the breadth of the distributions of light intensities of the two populations as well as their relative average intensities.

THE STRATEGY OF GATING

In flow cytometry the term *gating* is applied to the selection of cells (according to their fluorescence and/or scatter characteristics) that will be carried forward for further analysis. The process of gating corresponds to the decisions made by the microscopist about what particles in a field to include in a count of any particular type of cell. Gating is generally acknowledged to be one of the most powerful, but also one of the most problematic, aspects of flow cytometry (right up there with spectral compensation). It is problematic primarily because flow cytometrists like to think of their technique as objective and do not like to admit that much of flow cytometric analysis rests on the foundation of a few subjective initial decisions. The ideal strategy for gating should therefore move us toward two goals: First, gating needs to become as objective as possible, and second, flow cytometrists

need to recognize explicitly those aspects of gating that continue to require subjective decisions.

To continue with the example drawn from leukocyte techniques, gating has been employed effectively in the use of FSC and SSC characteristics for the selection of lymphocytes from within a mixed population of cells from peripheral blood. This use of gating is derived from two causes: First, a frequent question asked by immunologists (whether of a microscope or of a flow cytometer) concerns the distribution of lymphocytes into subpopulations. For example, “what percentage of lymphocytes are B lymphocytes?” or “what percentage of lymphocytes are CD8-positive lymphocytes?” Second, as discussed at the beginning of this chapter, lymphocytes possess physical characteristics that generally allow them to be distinguished from other types of leukocytes in both flow and microscopic analysis. Therefore, if one wants to know what percentage of the lymphocytes are CD8 positive, one simply defines the FSC and SSC characteristics of lymphocytes (by flow) or the nuclear and cytoplasmic patterns of lymphocytes (by microscope) and then analyzes the particles within this gate (i.e., with the defined characteristics) to see which of these stain with an anti-CD8 monoclonal antibody more intensely than an unstained control (Fig. 6.8). The ability to define a lymphocyte gate takes a bit of practice, but it is usually an easy decision for a skilled flow cytometrist (and also for a skilled cytologist using a microscope) when

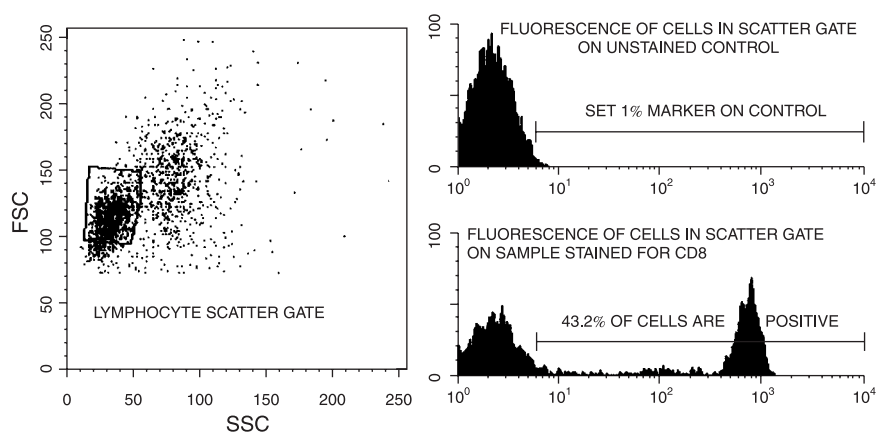


Fig. 6.8. A common type of flow analysis. The cells within a lymphocyte gate are analyzed, first in an unstained control sample and next in a stained sample, to determine how many cells within that gate are positively stained.

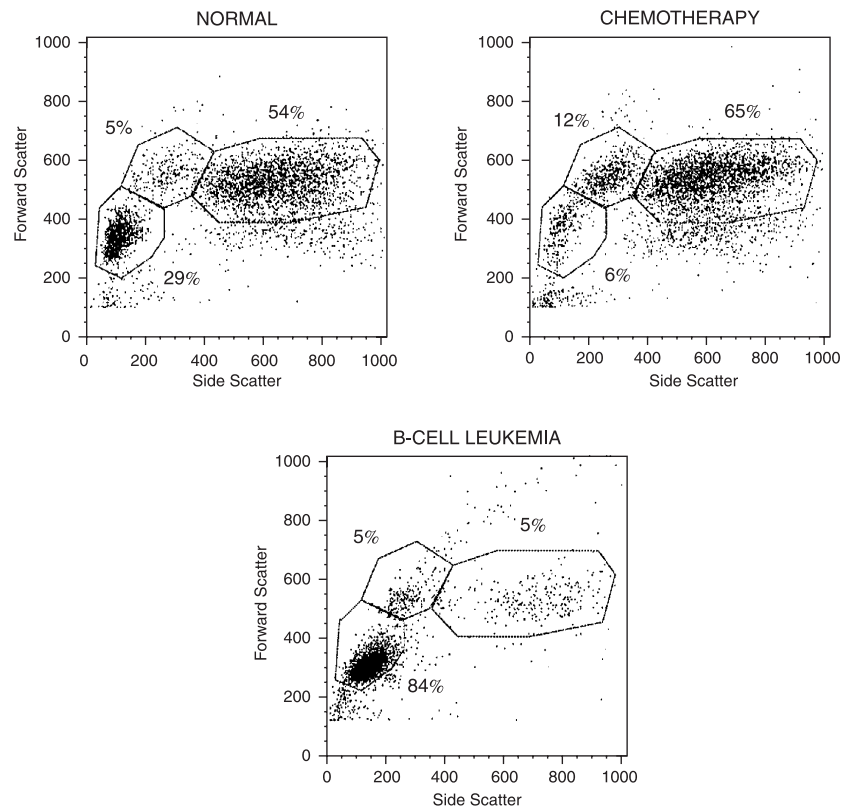


Fig. 6.9. Peripheral blood cells from a normal volunteer, a patient receiving chemotherapy, and a patient with B-cell leukemia. Normal cells appear as a tight cluster of lymphocytes with a diffuse group of monocytes on their shoulder possessing brighter FSC and SSC and a large cluster of cells with high SSC (neutrophils). The patient receiving chemotherapy has clusters in similar positions, but with far fewer lymphocytes that merge at their top end into the monocytes. The leukemic patient's cells are almost exclusively lymphocytes, which have slightly lower FSC than normal cells. Data files were provided by Marc Langweiler and Sharon Rich.

blood is relatively normal. It can, however, be a very difficult decision, by either technique, when blood is abnormal (e.g., blood from immunosuppressed patients, who have relatively few and possibly activated lymphocytes).

Figure 6.9 shows the light scatter profiles from cell preparations from a normal donor and from patients with leukemia and receiving chemotherapy, just to show diverse examples of light scatter profiles.

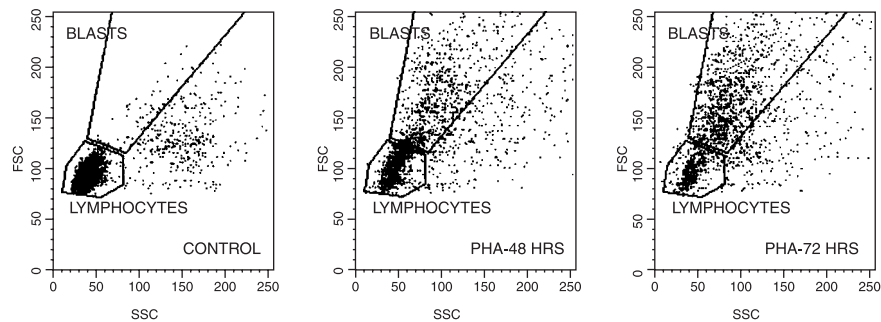


Fig. 6.10. The changes in patterns of scattered light that occur when lymphocytes become activated.

Figure 6.10 indicates the change in light scatter that occurs when lymphocytes become activated or stimulated to divide; both FSC and SSC intensities increase, and the lymphocytes “move out of the lymphocyte gate.” When few lymphocytes are present or those that are present have enlarged after immunological challenge, decisions about what is or is not a lymphocyte become difficult for both microscopists and cytometrists. The most important message here is that this type of decision, whether it be microscopic classification or a cytometrist’s gating, is essentially a subjective decision. It is based on training, skill, and practice. Its subjective nature can lead to different results from different operators, particularly when operators are inexperienced or when abnormal specimens are being analyzed, because the percentage of cells within a gate that are stained (with any given stain) will vary depending on the range of cells included within that gate (see Table 6.2). If we want to analyze lymphocytes, then the gate should ideally include all the lymphocytes from the sample and should include only lymphocytes.

Recognition of the need for some objectivity in gating decisions (both because operators may not be experienced and because not all samples are “normal”) has led to attempts both to automate the defining of the gate and to describe the goodness of the gate, once defined. Progress toward automation with lymphocytes brings us to the technique of back-gating, which has proved informative as a strategy for flow cytometric analysis in general. Historically, gating has been performed on the SSC and FSC characteristics of cells (by

TABLE 6.2. The Effect of Size of the FSC/SSC Gate on Determination of the Characteristics of Cells Within that Gate: Peripheral Blood Mononuclear Cells from a Heart/Lung Transplant Patient

	Small gate (691 cells)	Large gate (1074 cells)
Lymphocytes	646 cells	928 cells
	94% of gated cells	86% of gated cells
CD3 cells (T cells)	81% of gated cells	70% of gated cells
IL-2r ⁺ (activated) T cells	12% of T cells	16% of T cells
CD20 cells (B cells)	9% of gated cells	8% of gated cells
CD4 cells (helper T cells)	56% of gated cells	51% of gated cells
CD8 cells (cytotoxic T cells)	24% of gated cells	20% of gated cells
CD16 (NK cells)	8% of gated cells	11% of gated cells

analogy with a microscopist's use of physical criteria to classify cells), and this gate has then been used to inquire about the fluorescence properties of the gated cells. Back-gating is really just a different sort of gating—it reverses the usual protocol by using a stained sample, placing a gate around particles with certain fluorescence characteristics, and then asking what the physical characteristics (i.e., FSC and SSC) of these particles are. Once the scatter characteristics of a population have been ascertained in this way, the subsequent placing of the scatter gate can be done on the basis of rational and defined criteria.

For example, if one stains leukocytes with a PE-conjugated monoclonal antibody specific for the CD14 determinant (a monocyte marker) and puts a gate around the cells that are brightly fluorescent, it can be determined where these bright cells fall in a plot of FSC versus SSC (Fig. 6.11). As it turns out, most of the CD14-positive cells have moderate SSC and moderate FSC and lie in a cluster on the FSC/SSC dot plot. It also turns out, however, that there are a few CD14-positive cells that lie outside this region. We are now in a position to count the total number of CD14-positive cells in the sample; to draw what we think is an appropriate gate within the FSC/SSC plot; and finally to ask two questions that will tell us how good a gate we have drawn: (1) How many of the CD14-positive cells have we excluded by the drawing of that gate? (2) What percentage of the cells within the gate are not monocytes? Thus the technique of back-gating has allowed us to make an “educated guess” in drawing

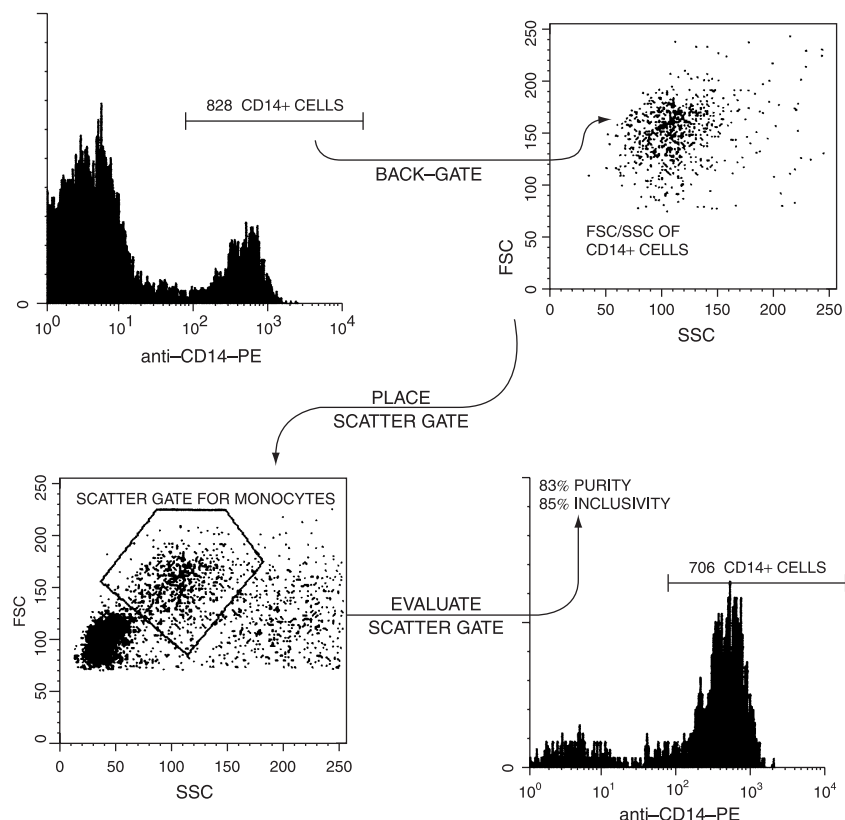


Fig. 6.11. Back-gating from CD14 fluorescence to determine the scatter characteristics from monocytes. Such back-gating facilitates the placing and then evaluation of a monocyte scatter gate.

a monocyte gate in the FSC/SSC plot; and it has then allowed us to evaluate that gate in terms of both purity and inclusivity. A perfect monocyte gate would contain only monocytes (100% purity) and would include all the monocytes (100% inclusivity). As we shall see, most gates are a compromise between these two goals. In any case, with back-gating to help us define a monocyte (FSC/SSC) gate, that scatter gate can be used in subsequent samples when staining the monocytes to analyze their phenotype.

By extension from this simple example, the technique of back-gating has been applied quite elegantly to leukocytes by the use of

a combination of two stains and two-color analysis. When a PE-conjugated monoclonal antibody specific for monocytes (as described above) is mixed with a fluorescein isothiocyanate (FITC)-conjugated antibody specific for a determinant found on all white cells (CD45 is a so-called leukocyte common antigen), this mixture can be used to stain a white cell preparation. After dividing the FITC/PE plot into four quadrants, it can be imagined that any erythrocytes or debris in the preparation will appear in the lower left quadrant (double negatives); any white cells will appear in the upper or lower right quadrants (FITC positive); and any monocytes will appear in the upper right quadrant (double positive). One might imagine that lymphocytes and granulocytes would appear in the lower right quadrant, as they are leukocytes but not monocytes (and should be FITC positive, PE negative).

The results from such a staining protocol are given in Figure 6.12. It turns out that they are even more useful than we might have imagined. It happens that granulocytes express a lower density of the common leukocyte antigen on their surface than do lymphocytes, and these two types of cells can be distinguished from each other as well as from the PE-positive monocytes in this staining mixture. Thus this protocol allows us to do the two things we have set as goals for our lymphocyte gating strategy. We can back-gate from the lymphocyte cluster in the FITC/PE plot to help us find the FSC/SSC region in which to draw our gate, and we can use the staining profile to evaluate that gate in terms of purity and inclusivity. In the case of the example shown in Figure 6.12, back-gating from the FITC-bright/PE-negative cluster leads us to a region for the FSC/SSC gating of lymphocytes. If we draw a reasonable scatter gate based on the scatter signals of the lymphocytes, we find that we have included most of the lymphocytes and that almost everything in that gate is a lymphocyte. A smaller gate might have higher purity but miss some lymphocytes; a larger gate might include 100% of the lymphocytes but some monocytes, neutrophils, and red blood cells as well. This kind of staining and back-gating protocol has led the way toward a fully automated gating procedure in which the gating and evaluation of that gate are done by computer. A computational gating algorithm can be written to adjust the scatter gate to aim for some desired level of purity and/or inclusivity.

However, any automated procedure will have some degree of

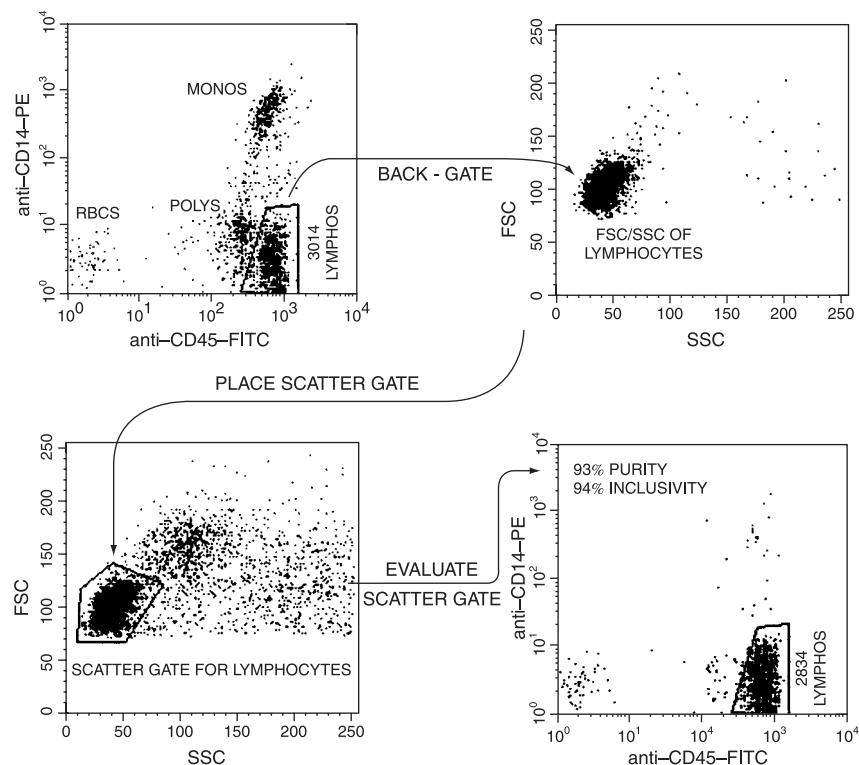


Fig. 6.12. Back-gating from CD14/CD45 fluorescence to determine the scatter characteristics of lymphocytes. Such back-gating facilitates the placing and then evaluation of a lymphocyte scatter gate within a peripheral blood mononuclear cell preparation.

difficulty with abnormal samples, and laboratories will tend to have their own (subjective) methods for compromising when the sample is such that a gate with both high purity and high inclusivity is not possible. By way of illustration of this situation, we can look at a sample of blood cells from an immunosuppressed patient (Fig. 6.13). Back-gating from the FITC-bright/PE-negative cluster leads us to placing a gate in a region of the FSC/SSC plot. We can see, however, that there appear to be lots of lymphocytes that have abnormally high FSC and SSC; these could be activated lymphocytes. If we draw a gate large enough to include these lymphocytes in our subsequent

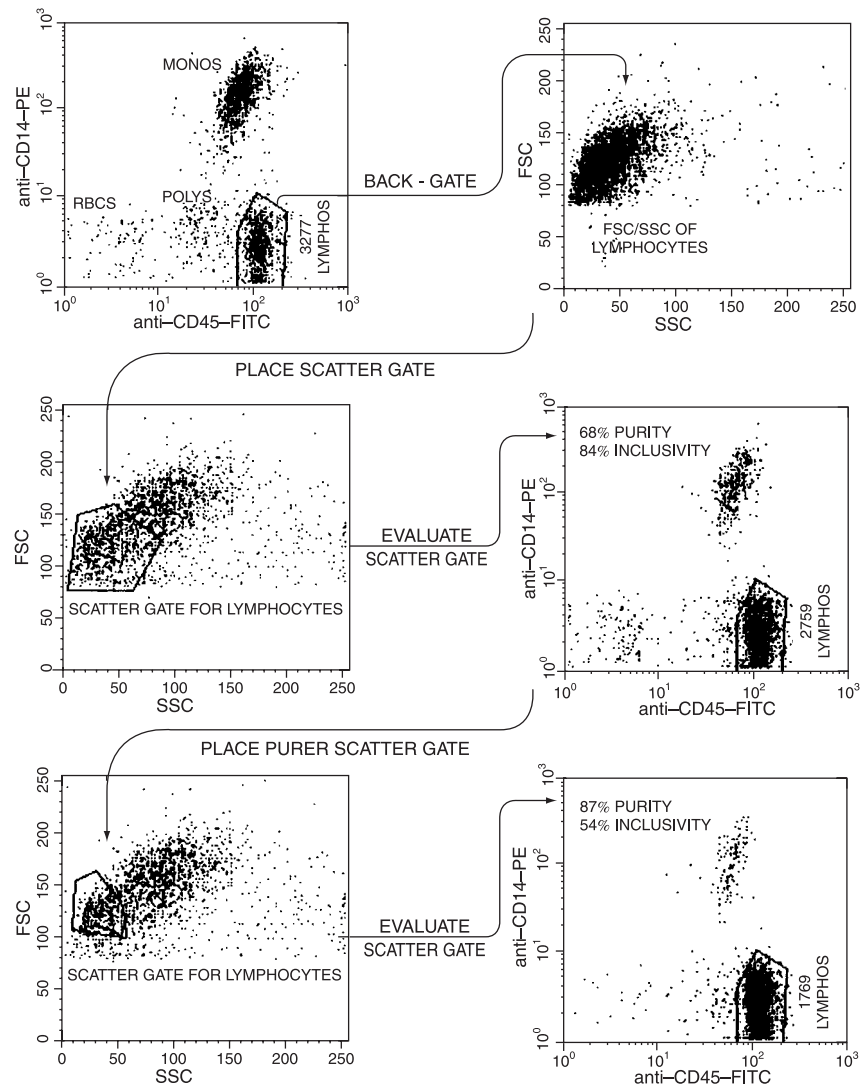


Fig. 6.13. Difficulties in placing a pure and inclusive lymphocyte scatter gate on a peripheral blood mononuclear cell preparation from a transplant patient with few and blasted lymphocytes.

analysis (high inclusivity), we find that the gate also includes many monocytes (low purity).

Unfortunately there is really no entirely satisfactory way out of this dilemma—the simple fact is that activated lymphocytes look, by flow cytometric scatter measurements, rather similar to monocytes (see Fig. 6.10). Any automated software will have trouble handling the type of situation when it is impossible to draw an FSC versus SSC gate with both high purity and high inclusivity. Practice will differ from lab to lab, with some operators tending to aim for high purity and accepting low inclusivity while others aim in the opposite direction. In either case, if one is expressing results as the percentage of lymphocytes that stain with a given marker, these results should be corrected for contamination by other particles (e.g., monocytes) within the lymphocyte gate. In fact, the situation is even more complicated than that. Because one may be staining lymphocytes for markers that appear on, for example, only activated cells (e.g., the interleukin-2 receptor), the inclusion or exclusion of the larger, more activated cells in the lymphocyte gate may have a profound effect on the result obtained even after this correction. Thus, the upshot is that we can aim for objectivity, but our decisions are often, by necessity, a subjective compromise between conflicting goals.

GATING ON FLUORESCENCE

As we have seen, one of the problems in gating lymphocytes according to their FSC and SSC characteristics is that it can be difficult, according to these scatter parameters, to distinguish lymphocytes from red blood cells, from platelets, from monocytes, and from debris. Immunologically activated patients may have lymphocytes that overlap monocytes; samples from some donors often have large numbers of red blood cells that are not easily removed by lysis or by centrifugation. Gating these preparations according to FSC and SSC may result in fluorescence analyses being reported as the stained percent, not of lymphocytes, but of an unknown mixture of cells.

For example, the more red blood cells that overlap lymphocytes in the scatter gate, the lower will be the percentage of those gated cells that are scored as CD4-positive (even though the percentage of lymphocytes that are CD4-positive may be identical in all cases). Now

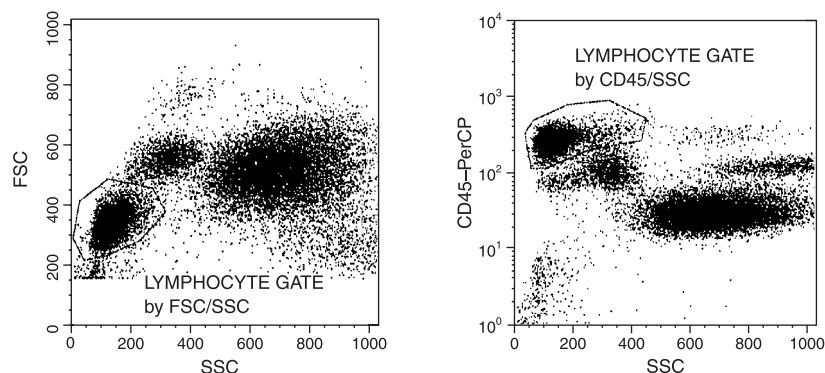


Fig. 6.14. The use of CD45 to exclude erythrocytes, platelets, and debris from a lymphocyte gate. The distinction between monocytes and lymphocytes can be ambiguous with either the FSC/SSC or the CD45/SSC gate. Data file provided by Marc Langweiler.

that many benchtop flow cytometers have “parameters to spare,” current clinical protocols for lymphocyte analysis suggest that antibodies against CD45 be added to all tubes as a third color to be used for gating. By gating on CD45 positivity along with SSC, the gated cells (Fig. 6.14) will not include high numbers of erythrocytes, debris, or platelets (all CD45 negative). In this way gating can be refined if you are using a flow cytometer with extra fluorescence parameters. The distinction between lymphocytes and monocytes is not, however, helped by this procedure (and the CD45/SSC gate in Fig. 6.14 indicates ambiguity here). Figure 6.15 illustrates CD45/SSC gating in a bone marrow sample, where this type of analysis has proved particularly useful in identifying clusters that are related to various mature and immature hematological lineages.

In fact, this CD45/SSC gating marks the general trend away from using scatter parameters toward a quite different strategy for flow analysis. The basic problem, as seen above, is that lymphocytes (or any other taxonomic group) are not a homogeneous collection of cells with perfectly delineated physical characteristics; they are mainly homogeneous, but they usually contain at least some cells at the fringes with marginal characteristics. Any gating based on FSC/SSC forces us either to exclude these fringe cells or to include many extraneous cells. With the availability of multicolor instrumentation

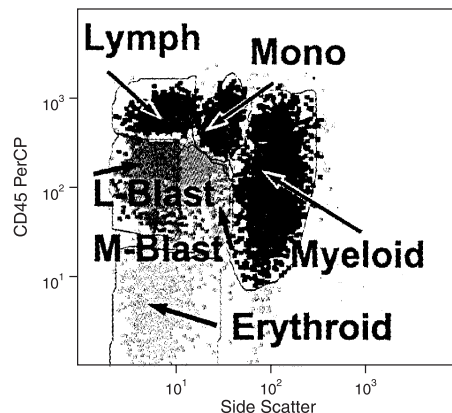


Fig. 6.15. Bone marrow from a normal donor showing CD45/SSC clusters. CD45 expression varies as cells mature. Lymph = lymphocytes; Mono = monocytes; L-Blast = lymphoblasts; M-Blast = myeloblasts; Myeloid = neutrophils; and Erythroid = cells of the erythroid lineage. From Loken and Wells (2000).

and multicolor stains, it has become possible to avoid making any of these difficult gating decisions and to include all cells in the analysis by using stain itself either to gate in or to gate out the cells of interest.

For example, if one is studying the prevalence of a particular subpopulation among lymphocytes, one could stain every peripheral blood mononuclear cell sample with a PE stain for monocytes in addition to an FITC conjugate of the marker of interest. Then, in the analysis stage, one could simply gate out (exclude) from analysis any PE-positive particles and analyze all the PE-negative particles for the percentage that are FITC-positive. Figure 6.16 shows an example of this protocol. By the use of three-color analysis, there are even greater possibilities. This type of analysis avoids the necessity of prior decisions about the FSC and SSC characteristics of the cells of choice (e.g., lymphocytes). It becomes particularly important when the cells of interest are less homogeneous in physical characteristics than lymphocytes. For example, by gating on a stain that is specific for cytokeratin (a protein found on tumor cells of epithelial origin), tumor cells within a mixed population from a breast tumor biopsy specimen can be selected for further analysis (e.g., DNA content) without any prejudgement about the FSC or SSC of a poorly defined and heterogeneous population of abnormal cells.

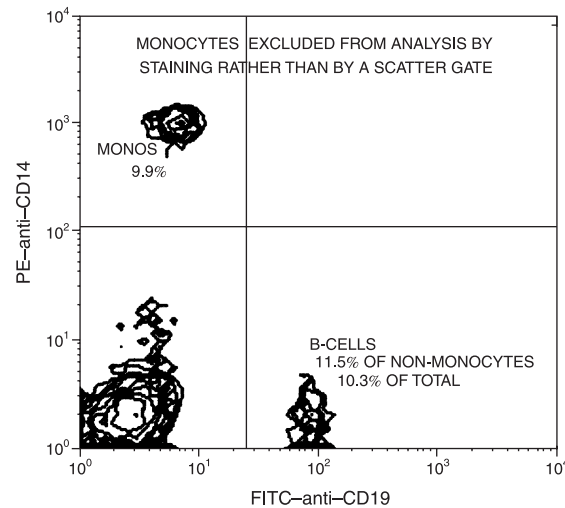


Fig. 6.16. Rather than a scatter gate, a PE-anti-CD14 stain can be used to exclude monocytes from a count of B and non-B lymphocytes. Data courtesy of Jane Calvert.

A logical extension of this kind of technique can be seen in the methodology proposed by PK Horan in 1986: No decisions based on the scatter characteristics of cells have been made. Cells are stained simply with a cocktail of conjugated monoclonal antibodies at appropriate (sometimes not saturating) concentrations, and all the cells in the mixture are classified according to their staining characteristics (Fig. 6.17). Staining cells from leukemic patients currently follows a similar strategy—where abnormal and normal cells are defined by the way they cluster in a two-dimensional dot plot. In other words, cells are defined by their relative intensities more than by their negativity or positivity for a given antibody.

At the beginning of this section, a strategy was described for placing a scatter (FSC/SSC) gate and then evaluating it in terms of purity and inclusivity. We were then forced to admit that gating is often an uneasy and subjective compromise between these conflicting criteria. We find therefore that we must conclude that using FSC and SSC characteristics to gate cells may not be a good thing after all. The availability of multicolor analysis has led to a trend toward using staining characteristics to define the cells of interest without regard to

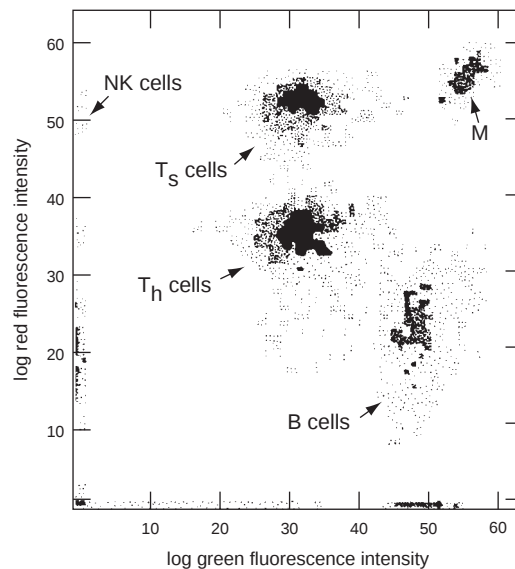


Fig. 6.17. The two-color fluorescence profile of peripheral blood mononuclear cells stained simultaneously with six different monoclonal antibodies to delineate five different populations of cells. From Horan et al. (1986).

their possibly variable physical (light scatter) properties. By using one or more colors in the analysis either to select or to exclude (gate in or gate out) particular groups of cells, we can avoid any prejudgement on the physical characteristics of those cells. This overall strategy makes sense when our system allows for multicolor analysis (with parameters to spare) and when antibodies are available to define the cells of interest. It is, nevertheless, still true that antibodies to define subsets of cells are not perfect: Cells of different phenotypes react with similar antibodies (sometimes, thankfully, at different intensities), and several antibodies are often required to fully define the taxonomy of a cell.

As a summary comment on gating, we need simply to remember that in flow cytometry our questions are usually formulated in terms of “what percentage of a certain population of cells is positive for a certain set of characteristics?” The choice of a gate defines that “certain population” of cells. The choice of that gate will therefore affect the answer to the question (the percentage positive). Whether gating

is applied by means of scatter characteristics, by means of staining characteristics, or not at all, the procedure still needs to be described and quantified if the results are to be meaningful and reproducible. It is only when we have stated exactly which “certain population of cells” we are analyzing that we have fulfilled our goals of objectivity and/or *explicit* subjectivity in flow analysis.

FURTHER READING

Chapters 3 and 5 in **Ormerod**, Chapters 3.2 and 3.3 in **Diamond and DeMaggio**, Chapters 10 and 11 in **Darzynkiewicz**, Chapter 6.2 in **Current Protocols in Cytometry**, and Chapters 17 and 34 in **Melamed et al.** are all good discussions of general lymphocyte staining methodology for flow analysis.

Chapter 1.3 in **Current Protocols in Cytometry** and Chapter 14 in **Darzynkiewicz** (both by Robert Hoffman) are excellent discussions of sensitivity and calibration.

Volume 33, number 2 (1998) of **Cytometry** is a special issue devoted to “Quantitative Fluorescence Cytometry: An Emerging Consensus.”